

## ORIGINAL ARTICLE

## Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex–amygdala activity

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Post-traumatic stress disorder (PTSD) and alcoholism are highly comorbid in humans and have partially overlapping symptomatic profiles. The aim of these studies was to examine the effects of traumatic stress (and stress reactivity) on alcohol-related behaviors and neuronal activation patterns. Male Wistar rats were trained to respond for alcohol, were exposed to predator odor (bobcat urine) paired with context and were tested for short- and long-term avoidance of the predator odor-paired context, alcohol self-administration and compulsivity of alcohol responding. Rats were re-exposed to the odor-paired context for western blot analysis of ERK phosphorylation in subregions of the medial prefrontal cortex (mPFC) and the amygdala. Rats that avoided the predator-paired chamber (Avoiders) exhibited persistent avoidance up to 6 weeks post conditioning. Avoiders exhibited increases in operant alcohol responding over weeks, as well as more compulsive-like responding for alcohol adulterated with quinine. Following re-exposure to the predator odor-paired context, Avoiders and Non-Avoiders exhibited unique patterns of neuronal activation in subregions of the mPFC and the amygdala, which were correlated with changes in avoidance and alcohol drinking. Furthermore, activity of upstream regions was differentially predictive of downstream regional activity in the Avoiders versus Non-Avoiders. An animal model for assessing the effect of traumatic stress on alcohol drinking reveals individual differences in neuronal activation patterns associated with re-exposure to traumatic stress-related stimuli, and may provide insight into the neural mechanisms underlying excessive alcohol consumption in humans with PTSD.

*Translational Psychiatry* (2013) 3, e296; doi:10.1038/tp.2013.70; published online 27 August 2013

**Keywords:** alcohol abuse; avoidance; escalation; individual differences; PTSD

## INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating disorder defined by the DSM-IV in terms of three major symptom clusters: persistent avoidance of trauma-related stimuli, hyperarousal and intrusive re-experiencing of the traumatic event.<sup>1</sup> Notably, PTSD occurs only in a subset of humans exposed to a traumatic stressor, estimated to be between 10 and 25% of individuals, depending on the stress stimulus and the criteria used to assess PTSD (for example, Breslau *et al.*<sup>2</sup>). Important for the present report, PTSD and alcohol-use disorders are highly comorbid in humans and have partially overlapping symptomatic profiles (for example, Engdahl *et al.*<sup>3</sup>).

PTSD has been described in terms of co-occurring maladaptive fear conditioning processes (that is, excessive fear learning or deficient fear extinction) and sensitized stress responses,<sup>4</sup> which are mediated by changes in partially overlapping neural circuits (for example, Pape and Pare,<sup>5</sup> and Shin and Liberzon<sup>6</sup>). Studies attempt to model these pathological neural cascades by exposing animals to a stimulus that mimics traumatic stress exposure in humans (for reviews, see Cohen *et al.*<sup>7</sup> and Logrip *et al.*<sup>8</sup>), although different stressors (for example, social defeat, footshock and predator odor) elicit different sets of behavioral and biological responses in rats.<sup>9</sup> Animals can be divided into subgroups based on behavioral responses to the stressor, as long as the behavioral response is not maximal or uniform across rats. Predator odor stress allows for the division of animals into subgroups that exhibit

either multiple co-occurring signs of behavioral dysregulation (for example, hyperarousal and high anxiety) or collective absence of these signs.<sup>10</sup> This clustering of behavioral changes suggests a common underlying etiology for multiple behavioral counter-adaptations in animals that do not cope well with a traumatic stressor and in humans with PTSD.<sup>11</sup>

Humans with pre-existing PTSD are more likely to develop alcohol dependence than non-PTSD controls.<sup>12</sup> PTSD and alcohol dependence are both defined by negative affective states (for example, hyperarousal and dysphoria) that result from maladaptive physiological responses to environmental stimuli.<sup>13,14</sup> Furthermore, an accumulation of evidence points to dysregulation of overlapping neural circuits following exposure to traumatic stress<sup>5</sup> and the transition to alcohol dependence.<sup>15,16</sup> The high rate of comorbidity and similar symptomatology of these two disorders in humans, as well as the recruitment of brain stress systems in both disorders, support a self-medication hypothesis in which affected individuals may consume excessive amounts of alcohol to ameliorate PTSD symptoms. Importantly, past studies have not explicitly assessed alcohol- and drug-taking behaviors in rodents that do and do not exhibit a PTSD-like state following exposure to a traumatic stressor.<sup>8</sup>

Animal models of PTSD-like behavioral symptoms can be used to dissect neural perturbations produced by maladaptive responses to traumatic events.<sup>17</sup> In this study, we assessed neuronal signaling patterns by measuring ERK phosphorylation

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Received 24 April 2013; revised 5 June 2013; accepted 15 July 2013

following exposure to a context previously paired with traumatic stress, mimicking symptom provocation studies in human PTSD patients. The ERK pathway is an intracellular signal transduction cascade that regulates synaptic plasticity in several limbic brain regions via its ability to relay extracellular signals into nuclear responses,<sup>18</sup> and ERK phosphorylation has been widely used as a measure of stimulus-driven neuronal activation.<sup>19,20</sup>

Here, we demonstrate that rats exhibiting persistent high avoidance of trauma-related stimuli also exhibit long-lasting post-stress increases in alcohol drinking, that ERK phosphorylation patterns are predictive of behavioral dysregulation in individual rats, and that high-avoidance, high-drinking rats exhibit altered neuronal activation patterns in the prefrontal cortex and amygdala, as well as a potential modification of connectivity between these regions.

## MATERIALS AND METHODS

### Ethics statement

All experiments were conducted in accordance with the institutional (LSUHSC, IACUC 2908; TSRI, IACUC 08-0015) and NIH guidelines.

### General methods

**Subjects.** Adult male Wistar rats were used in all experiments. All experiments were performed during the dark period (0800–2000 h) of a 12 h:12 h cycle. In all cases, rats were experimentally naïve at the start of experiments, and were habituated to housing conditions and experimenter handling for ≥1 week before the start of experimental manipulations.

### Behavioral procedures

**Alcohol self-administration (Experiments 1 and 2):** Rats were trained to respond for alcohol (10% w/v) and water in a concurrent, two-lever, free-choice operant situation. Volumes of 0.1 ml each of alcohol and water were delivered on a fixed ratio-1 schedule. Before the start of operant training, rats were given access to 10% w/v alcohol and water in the home cage for a single 24-h period. Intakes were not recorded and the purpose of this access was to avoid neophobic response to alcohol in operant chambers. On the first day of operant training, rats were given 12 h access to a single lever (right lever) that produced 0.1 ml deliveries of water on a fixed ratio-1 schedule with *ad libitum* food available on the floor of the operant chamber. On the second day of operant training, rats were given 2 h access to two levers that produced 0.1 ml deliveries of alcohol versus water on fixed ratio-1 schedules. During the second session and all subsequent sessions going forward, alcohol was paired with the right lever and water was paired with the left lever. On the third day of operant training, rats were given 1 h access to the two-lever contingency, and starting on the fourth day of training and for all subsequent sessions going forward rats were given 30 min access to the two-lever alcohol versus water contingency. This procedure allows rats to learn to press for alcohol in an operant situation without the use of sweeteners or fluid deprivation. Rats were allowed ~15 sessions (see design of each experiment below) of operant responding for alcohol versus water until operant responding was stable and reliable for these rats, at which point they were divided into two groups matched for intakes across the final 5 days of this baseline period: rats to be exposed to traumatic (predator odor) stress and rats not to be exposed.

**Aversive contextual conditioning (Experiments 1–3):** Rats were exposed to predator odor versus no odor (or no odor vs no odor for controls) in two separate contexts that differed on both visual (wall patterns) and tactile (floor grids) cues following stabilization of operant alcohol responding in Experiments 1 and 2. On the first day of this procedure, rats were allowed 5 min of free exploration of the conditioning apparatus, which consisted of two conditioning chambers (36 cm length × 30 cm width × 34 cm height) connected by a small triangular compartment. The time that rats spent in the two conditioning chambers was recorded as the pre-conditioning baseline. The details of pre-conditioning baseline compartment times and assignment of unconditioned stimuli to compartments is detailed below in the Experiment-Specific Procedures section for Experiments 1–3.

On the day following the pre-conditioning preference test, rats were placed in one of the two contexts for 15 min in the absence of odor, then

they were returned to the home cage. On the third day of the procedure, rats were placed in the other context for 15 min and a sponge soaked in bobcat urine (or no odor for controls) was placed in the room. On the fourth and final day of this procedure (24 h post-odor exposure), rats were again allowed 5 min of free exploration of the conditioning apparatus. For all experiments, rats in the control group were never exposed to bobcat urine for the duration of the experiment.

Avoidance of predator-paired context was quantified as post-conditioning time in predator context minus pre-conditioning time in predator context. Rats that exhibited >10 s decrease in time spent in predator-paired chamber following conditioning (relative to baseline) were termed highly reactive and will be called Avoiders from here on in this study, whereas rats that exhibited <10 s decrease in time spent in predator-paired chamber following conditioning (relative to baseline) will be called Non-Avoiders. This group division criterion (> or < 10-s decrease) was chosen, because in our experiments not many animals exhibited avoidance scores approximately equal to –10 s (providing an unambiguous distinction between groups). In addition, we define avoidance as the presence of a behavior and non-avoidance as the absence of that behavior (rather than the presence of another behavior); therefore, we decided not to classify animals that exhibited very small reductions (<10 s) in time spent in predator odor-paired chamber as Avoiders. Finally, we intentionally chose a criterion for groups that represented a data point that would remain constant across experiments (that is, time spent in predator odor-paired context) rather than a cohort-specific criterion (for example, median split) that was likely to change across experiments. This division criterion is at least partly validated by its predictive value for subsequent changes in brain and behavior, as described in the Results section. The percentages of animals designated as Avoiders versus Non-Avoiders are detailed below in the Experiment-Specific Procedures section for Experiments 1–3.

We modeled traumatic stress in humans by exposing rats to predator odor for the following reasons: (1) the effects of other more commonly used stressors (for example, restraint and footshock) on alcohol drinking are transient, variable and inconsistent across studies and time,<sup>8,21</sup> (2) to avoid using a stressor that administers physical pain to the rat and (3) the perceived presence of a predator has powerful long-lasting behavioral and biological effects across species.<sup>22</sup> Predator stress models also lend themselves particularly well to the detection of individual differences,<sup>7</sup> a critical component of animal models of the PTSD symptomatology.

**Appetitive contextual conditioning (Experiment 3):** In this procedure, rats were allowed to consume a sweet solution (supersac, 3% glucose, 0.125% saccharin;<sup>23</sup>) or water on alternating days in two separate contexts that differed on both visual (wall patterns) and tactile (floor grids) cues. On the first day of this procedure, rats were allowed 5 min of free exploration of the conditioning apparatus, which consisted of two conditioning chambers connected by a small triangular compartment. The time that rats spent in the two compartments was recorded as the pre-conditioning baseline, and rats were assigned supersac solution in one compartment and water solution in the other compartment. On the day following the pre-conditioning preference test, rats were placed in one of the two contexts for 15 min and were allowed to drink water *ad libitum* from a graduated bottle, then they were returned to the home cage. On the third day of the procedure, rats were placed in the other context for 15 min and were allowed to drink supersac *ad libitum* from a graduated bottle, then they were returned to the home cage. The procedure for days 2 and 3 was repeated twice more (days 4–7), such that rats received water-context pairings for 3 total days and supersac context pairings for 3 total days. On day 8, rats were allowed 5 min of free exploration of the conditioning apparatus. Preference for supersac-paired context was quantified as post-conditioning time in supersac context minus pre-conditioning time in supersac context.

**Compulsivity of alcohol self-administration (Experiment 1):** Rats that had previously been trained to self-administer alcohol were allowed to respond for 10% w/v alcohol adulterated with gradually increasing concentrations of quinine, as previously described.<sup>24</sup> Briefly, rats were allowed to respond for ethanol/quinine solutions versus water during a 30-min operant self-administration over 7 consecutive days. On the first day of this procedure, 0% quinine was added to the ethanol solution, and quinine concentrations on following days were 0.0005, 0.001, 0.0025, 0.005, 0.01 and 0.025% quinine. Operant response data are presented as percent change from baseline (10% w/v ethanol + 0% quinine) for each rat.

in unstressed controls) and were killed, and the brains were removed for western blot analysis.

**Experiment 2.** The purpose of Experiment 2 was to confirm Experiment 1 findings of post-stress escalation of alcohol drinking in Avoider rats, and also to test animals for a longer period of time (19 days instead of 11 days) to assess the persistence of this effect. Rats ( $n=29$ ) were trained to self-administer 10% w/v ethanol versus water in a two-lever operant situation during 30-min self-administration sessions, as described above, for a period of 18 days. Rats then underwent the 4-day aversive contextual conditioning procedure described above. To confirm that the Experiment 1 results were not due to the biased conditioning design, rats in the experimental group were assigned predator odor exposure in an unbiased design such that pre-conditioning preference for predator and neutral contexts was counterbalanced (baseline times = 133 vs 136 s in the two contexts subsequently paired with predator odor or no odor, respectively). Following the 4-day aversive conditioning procedure, rats were once again allowed to self-administer 10% w/v ethanol versus water on days 2, 8, 12, 15 and 19 following exposure to predator odor (or no odor in controls).

**Experiment 3.** The purpose of Experiment 3 was to determine whether innate anxiety-like behavior or the ability to classically condition an appetitive stimulus (to assess learning ability of subsequent Avoider and Non-Avoider groups) was predictive of subsequent avoidance behavior. One week following vivarium arrival, rats ( $n=20$ ) were tested for innate anxiety-like behavior on the EPM, as described above. Four days following the EPM test, rats were allowed to explore an apparatus with three chambers that differ in the visual (wall pattern) and tactile (floor composition) cues. For each rat, the three compartments were assigned one of the three unconditioned stimuli: supersac solution, predator odor and water solution/no odor (that is, neutral context). Using two of these three compartments, rats underwent the 8-day appetitive contextual conditioning procedure as described above. Then, 3 days following the post-conditioning preference test for the supersac-paired context, rats underwent the 4-day aversive contextual conditioning procedure described above (using the same neutral context and the third previously unused compartment for predator odor exposure). Rats in this experiment were not divided into Avoider and Non-Avoider groups; instead, the variability of conditioned preference and aversion times were analyzed to assess whether predator odor-paired context aversion might be attributable to the rats' ability to classically condition contextual pairings with unconditioned stimuli. To facilitate conditioning and the variability of conditioning, a biased design was used for this experiment (in a three-chamber pre-test, baseline times = 122 s for predator odor-paired chamber, 95 s for neutral chamber and 62 s for supersac-paired chamber).

**Statistical analysis.** Data are reported as mean  $\pm$  s.e.m. Data were analyzed with one-way, two-way and three-way analysis of variance (ANOVA) as described in text, and significant group differences were probed with a Student-Newman-Keuls or Bonferroni *post-hoc* analysis. Pearson's correlations were used to determine relationships between behavioral and molecular outcomes. In all cases, statistical significance was set at the  $P < 0.05$  level. Where appropriate, data in figures are presented as difference scores relative to baseline or percent change from baseline.

## RESULTS

Traumatic stress produces persistent avoidance in some but not all rats

Figure 1a shows that Avoiders exhibited significantly greater avoidance of the predator-paired chamber at 24 h and 6 weeks post-predator odor exposure,  $F(1,35) = 29.49$ ,  $P < 0.001$ , relative to Non-Avoiders. Avoidance of the predator odor-paired chamber at 24 h was positively and significantly correlated with the avoidance of the same chamber 6 weeks later,  $r(35) = 0.37$ ,  $P = 0.025$ . In animals trained to self-administer alcohol and then exposed to the 4-day aversive conditioning procedure, 59% of rats (61% in Experiment 1; 55% in Experiment 2) were designated as Avoiders according to the criteria outlined above. It is worth noting that the proportion of animals that met Avoider criteria was similar in the two experiments even though Experiment 1 utilized a biased conditioning design and Experiment 2 utilized an unbiased conditioning design.

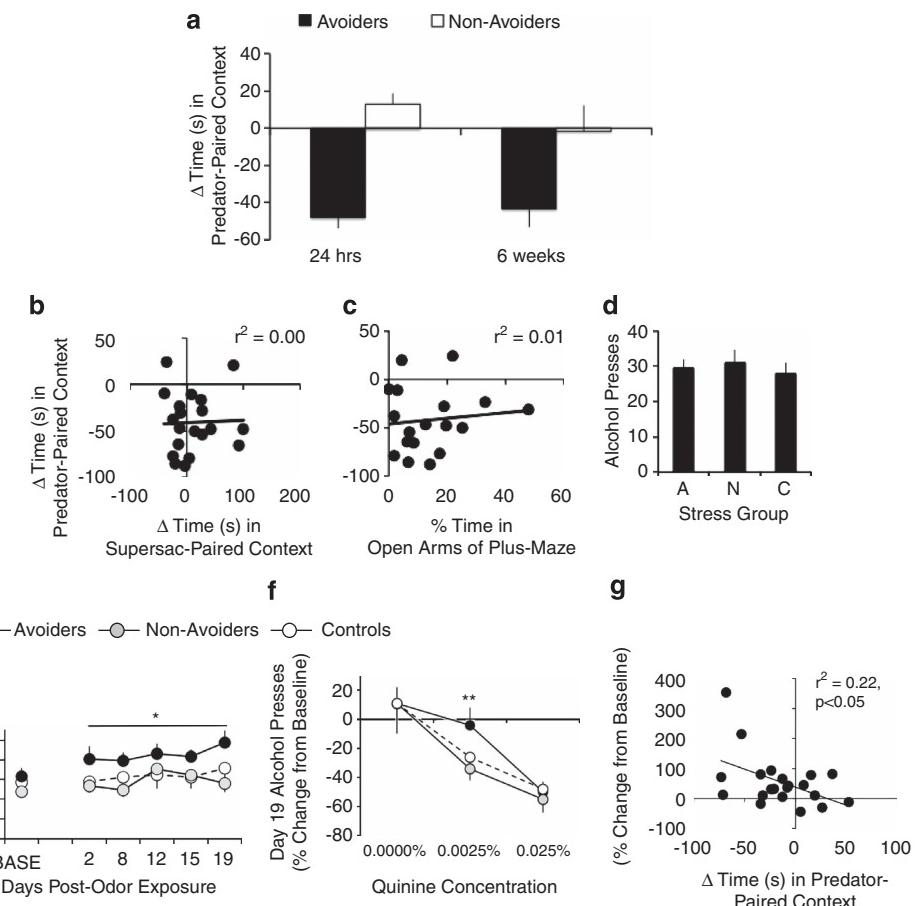
**Elevated plus-maze (Experiment 3):** The elevated plus-maze (EPM) test was used to test anxiety-like behavior in conditions previously described.<sup>25</sup> Briefly, the maze was made of black Plexiglas and consisted of four arms (50 cm long  $\times$  10 cm wide); two closed arms had 40-cm high, dark walls and two open arms had no walls. The height of the apparatus was 50 cm from the floor. During testing, open arms received 22–350 lux and closed arms received <1 lux of illumination. These lighting conditions produce % open arm times of 15–20% in our lab, consistent with the literature for EPM results observed in genetically heterogeneous rats (in results reported below, mean % open arm time is approximately equal to 14%). Individual rats were placed in the center of the maze facing a closed arm and were removed after 5 min. Behavior was recorded by a digital camera and was scored by an experimenter blind to treatment conditions. The apparatus was cleaned between subjects. Primary measures were percent open arm time (open/open + closed) and number of closed-arm entries (defined as all four paws entering).

**Western blot analysis.** Individual ERK phosphorylation levels in the brain regional homogenates were determined as described in Edwards et al.<sup>20</sup> Immediately upon completion of the final 15-min context exposure (see below), animals were killed under light isoflurane anesthesia by decapitation. Brains were rapidly dissected and snap frozen in isopentane. Regional tissue samples were obtained using 12–16 gauge punches from frozen coronal brain slices (0.5 mm thick) obtained by the use of a cryostat. Tissue samples were homogenized by sonication in lysis buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA and 1% SDS, with protease inhibitor cocktail and phosphatase inhibitor cocktails II and III diluted 1:100; Sigma, St Louis, MO, USA), heated at 100 °C for 5 min and were stored at –80 °C until the determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA, USA). Samples of protein (15 µg) were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels by using a Tris/Glycine/SDS buffer system (Bio-Rad), followed by electrophoretic transfer to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked overnight in 5% non-fat milk at 4 °C and were then incubated in primary antibody recognizing the dual phosphorylated form of ERK (1:1000, 5% non-fat milk; Cell Signaling, Danvers, MA, USA). Membranes were washed and labeled with species-specific peroxidase-conjugated secondary antibody (1:10 K; Bio-Rad) for 1 h at room temperature. Following chemiluminescence detection (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA), blots were stripped for 20 min at room temperature (Restore; Thermo Scientific) and were reprobed for total protein levels of ERK (1:10 K; Cell Signaling). Immunoreactivity was quantified by densitometry (ImageJ 1.45S; NIH) under linear exposure conditions. Separate gel sets composed of control and experimental (Avoider and Non-Avoider) animals were processed in parallel for each region. Densitized values were expressed as a percentage of the mean of control values for each gel to normalize data across blots. Individual phosphoprotein levels were normalized to individual total protein levels to generate ERK phosphorylation (pERK)/ERK ratio values for statistical comparison.

## Experiment-specific procedures

**Experiment 1.** The purpose of Experiment 1 was three-fold: (1) to assess alcohol drinking and compulsion of alcohol drinking in rats exposed to predator odor stress, (2) to assess the persistence of avoidance behavior and the predictive value of avoidance for subsequent changes in alcohol drinking, and (3) to generate tissue from these groups of rats to assess neural correlates of behavioral changes.

Rats ( $n=48$ ) were trained to self-administer 10% w/v ethanol versus water in a two-lever operant situation during 30-min self-administration sessions, as described above, for a period of 18 days. Rats then underwent the 4-day aversive contextual conditioning procedure described above. For rats in the experimental group, predator odor exposure was assigned to compartments in a biased design (baseline time for predator odor-paired chamber = 145 s and baseline time for neutral chamber = 94 s). Following the 4-day aversive conditioning procedure, rats were once again allowed to self-administer 10% w/v ethanol versus water on days 2, 5, 8 and 11 following exposure to predator odor (or no odor in controls). Six weeks following exposure to predator odor, rats were again tested for avoidance of the predator-paired chamber. Finally, rats were tested for compulsion of alcohol responding using the quinine adulteration procedure described above. At the end of behavioral experiments, all rats were left in the home cage undisturbed for a period of 1 week, at which point they were given one final 15-min exposure to the predator-paired context (neutral context



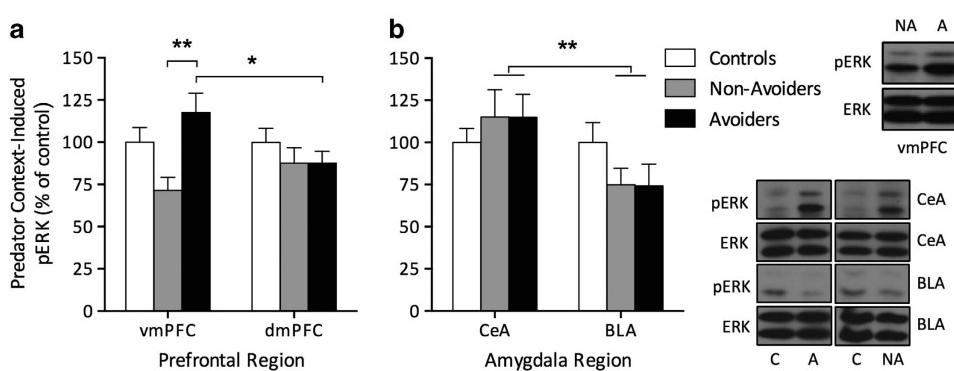
**Figure 1.** (a) Mean ( $\pm$  s.e.m.) change in time (s) spent in predator-paired context from pre-conditioning test to post-conditioning test, quantified as post-test score minus pre-test score. Rats (Experiment 1) were divided into Avoiders ( $n = 23$ ) and Non-Avoiders ( $n = 14$ ) based on avoidance of predator-paired chamber at 24 h post conditioning, and then were tested again for avoidance 6 weeks later. Rats that exhibited high avoidance of the predator-paired chamber at 24 h post-conditioning also exhibited avoidance 6 weeks later. (b) Scatter plot for individual rat (Experiment 3) shows change in preference for a context repeatedly paired with an appetitive stimulus (saccharin + glucose solution) versus change in preference for a context repeatedly paired with predator odor. There was no predictive value by the degree to which a rat conditioned the appetitive stimulus for the degree to which a rat conditioned the aversive stimulus. This also suggests that differences in avoidance are not attributable to differences in ability of rats to learn. (c) Scatter plot for individual rat (Experiment 3) shows percent time spent in open arms of EPM (5-min test) in experimentally naive rats (index of innate anxiety-like behavior) versus change in preference for a context repeatedly paired with predator odor. There was no predictive value by innate anxiety-like behavior for the degree to which a rat conditioned the aversive stimulus. (d) Mean operant alcohol responses ( $\pm$  s.e.m.) per 30 min session for Avoider, Non-Avoider and non-stressed control groups (Experiments 1 and 2 combined) across the last 3 days of the pre-conditioning baseline self-administration period. Groups did not differ in baseline alcohol self-administration. These data also provide further support for the notion that differences in avoidance are not attributable to differences in ability of rats to learn. (e) Mean ( $\pm$  s.e.m.) operant presses for alcohol per 30-min self-administration session by rats in Experiment 2 (results similar in Experiment 1, see text) during intermittent testing across the 19 days following exposure to predator odor (Avoider and Non-Avoider groups) or no odor (control group). Avoider rats exhibited persistent increases in operant alcohol responding relative to the two other groups and their own baseline across the 19 days following exposure to odor. \* $P < 0.05$  significant main effect of group. Avoiders exhibited marginally non-significant increases in alcohol responding relative to Non-Avoiders ( $P = 0.06$ ) and unstressed Controls ( $P = 0.055$ ). (f) Mean ( $\pm$  s.e.m.) self-administration of alcohol solution adulterated with progressively increasing quantities of quinine, quantified as percent change from baseline (10% w/v ethanol plus 0% quinine) for each rat (Experiment 1). A 10  $\times$  higher concentration (0.025% vs 0.0025%) of quinine was required to reduce operant alcohol self-administration in Avoider rats vs Non-Avoider and Control rats, indicative of more compulsive-like alcohol drinking in Avoider rats. \*\* $P < 0.001$  Avoiders responded significantly more for alcohol + 0.0025% quinine relative to unstressed Controls. There was a non-significant tendency of Avoiders to press more for this solution than Non-Avoiders ( $P = 0.10$ ). (g) Scatter plot for individual rat (Experiment 2) shows change in preference for predator-paired context versus percent change in operant alcohol responding at day 19 post-odor exposure relative to baseline. Rats that exhibited high avoidance of the predator-paired context 24 h post-odor exposure self-administered more alcohol at 19 days post-odor exposure.

Figures 1b-d show the results of several behavioral measures that failed to predict avoidance of predator-paired chamber in individual rats. The conditioned place preference exhibited by rats for a supersac-paired context did not predict avoidance of a predator odor-paired context,  $r = 0.03$ ,  $P > 0.05$  (Figure 1b). Time spent in the open arms of the EPM also did not predict the avoidance of a predator odor-paired context,  $r = 0.11$ ,  $P > 0.05$  (Figure 1c). Baseline alcohol drinking (pooled across rats in Experiments 1 and 2) did not differ between control rats and rats that exhibited high or low avoidance of predator odor-paired

context,  $F(2,75) = 0.23$ ,  $P > 0.05$ , suggesting that limited-access operant alcohol self-administration training before odor exposure does not make rats more or less likely to exhibit high reactivity to a traumatic stress.

#### Avoiders exhibit excessive alcohol self-administration

In Experiments 1 and 2, rats that exhibited high avoidance of the predator odor-paired chamber 24 h following exposure also exhibited lasting increases in operant alcohol self-administration



**Figure 2.** (a) ERK phosphorylation (pERK) in the medial prefrontal cortex (PFC) after re-exposure (15 min) to the predator odor-paired context. Relative to neutral-paired animals (controls), re-exposure to the context produced a bidirectional regulation of pERK levels in the vmPFC in the odor-paired groups, with Avoiders displaying relatively higher ERK phosphorylation compared with that of Non-Avoiders. This relationship was not observed in the dorsomedial PFC (dmPFC). \*\* $P < 0.01$  higher ERK phosphorylation levels in Avoiders versus Non-Avoiders or \* $P < 0.05$  higher ERK phosphorylation levels in vmPFC versus dmPFC in Avoiders. (b) ERK phosphorylation in the amygdala subregions after re-exposure to the odor-paired context. As a percentage of neutral-paired control animals, Avoider and Non-Avoider stress-paired groups displayed more ERK phosphorylation in the central nucleus of the amygdala (CeA) versus basolateral amygdala (BLA). \*\* $P < 0.01$  higher ERK phosphorylation levels in the CeA versus BLA of pooled stress-paired groups by analysis of variance (main effect of group). Data reflect the mean  $\pm$  s.e.m. of pERK/ERK ratios and are expressed as a percentage of neutral-paired controls. Inset: representative western blot images for pERK and ERK levels in vmPFC, CeA and BLA in Control (C), Avoider (A) and Non-Avoider (NA) groups.

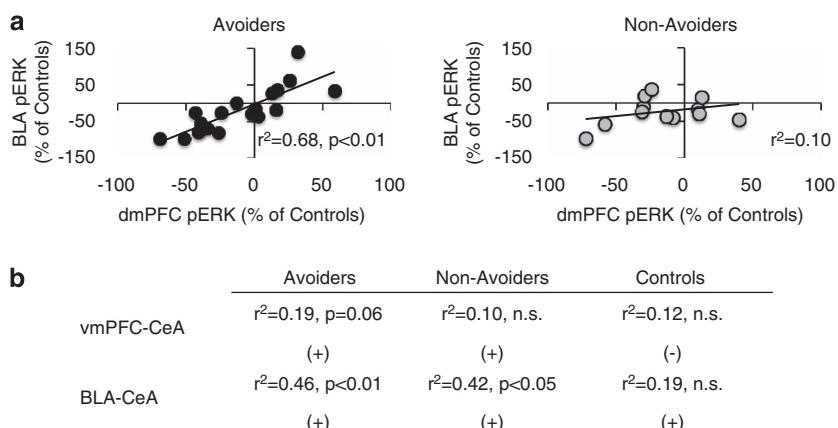
relative to Non-Avoiders and unstressed controls. A two-way (group  $\times$  days) repeated measures ANOVA of alcohol lever presses from the 11 days following stress exposure in Experiment 1 (data not shown) yielded a significant main effect of the group,  $F(2,45) = 4.55$ ,  $P = 0.016$ , and post-hoc analyses revealed that Avoiders exhibited significant post-stress increases in operant alcohol responding relative to both Non-Avoiders ( $P = 0.015$ ) and unstressed controls ( $P = 0.05$ ). A two-way (group  $\times$  days) repeated measures ANOVA of alcohol lever presses from the 19 days following stress exposure in Experiment 2 (Figure 1e) yielded a significant main effect of the group,  $F(2,26) = 3.47$ ,  $P = 0.046$ , and post-hoc analyses revealed that Avoiders exhibited marginally non-significant increases in alcohol responding relative to Non-Avoiders ( $P = 0.06$ ) and unstressed controls ( $P = 0.055$ ). Increases in alcohol drinking appeared to incubate with time in Avoiders, as evidenced by the fact that Avoider rats exhibited peak alcohol responding 19 days post stress. Importantly, high avoidance of the predator odor-paired context by exposed rats was predictive of increased alcohol drinking 19 days post stress,  $r = -0.47$ ,  $P = 0.03$ , suggesting that high stress reactivity 24 h post stress (as measured by conditioned place aversion) produces long-lasting neural changes that promote excessive alcohol drinking (Figure 1g).

To test compulsivity (that is, persistence) of alcohol responding when alcohol is paired with an aversive stimulus, rats were tested on consecutive days for operant responding for alcohol adulterated with progressively increasing quantities of quinine. As illustrated in Figure 1f, a 10  $\times$  higher concentration of quinine (0.025% vs 0.0025%) was required to reduce operant alcohol responding in Avoider rats relative to Non-Avoider rats and unstressed controls. A two-way (group  $\times$  concentration) repeated measures ANOVA of lever presses for alcohol adulterated with 0.0025% versus 0.025% quinine revealed significant main effects of the group,  $F(2,43) = 5.10$ ,  $P = 0.01$ , and the concentration,  $F(1,43) = 33.37$ ,  $P < 0.001$ , as well as a significant group  $\times$  concentration interaction effect,  $F(2,43) = 3.32$ ,  $P < 0.05$ . Post-hoc analysis of alcohol + 0.0025% (moderate concentration) quinine data revealed that Avoiders responded significantly more for this solution relative to unstressed controls ( $P < 0.001$ ); there was a non-significant tendency of Avoiders to press more for this solution than Non-Avoiders ( $P = 0.10$ ). In contrast, none of the

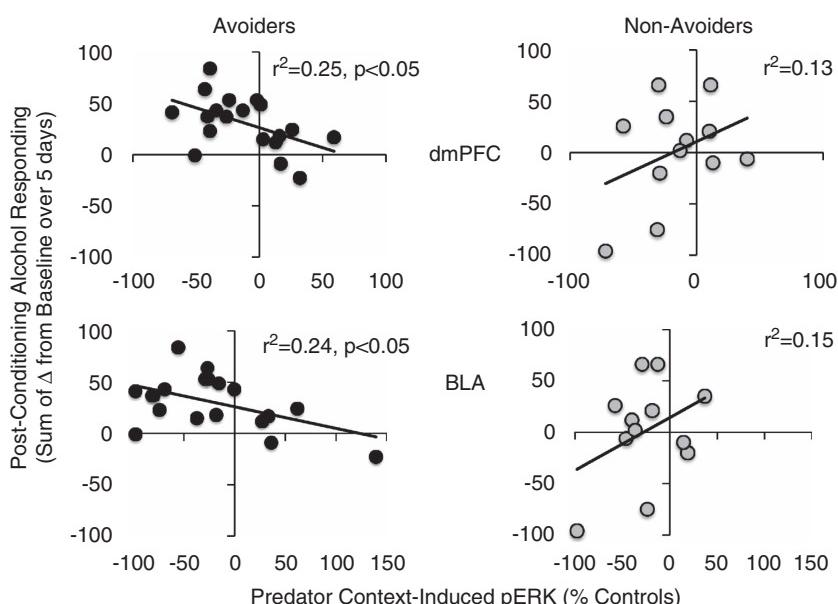
groups differed in their pressing for the alcohol solution adulterated with the highest quinine concentration (0.025%).

#### Traumatic stress drives neuronal activation according to avoidance behavior

At the end of behavioral testing, animals were exposed to a final 15-min exposure to the predator-paired context (neutral context in unstressed controls) and were killed, and the brains were removed for western blot analysis to measure context-associated ERK phosphorylation. As illustrated in Figure 2a, a two-way (group  $\times$  medial prefrontal cortex (mPFC) region) ANOVA revealed a significant interaction effect on pERK levels,  $F(2,98) = 3.25$ ,  $P < 0.05$ , and post-hoc analyses indicated that pERK levels in ventromedial PFC (vmPFC) were significantly higher in Avoiders relative to Non-Avoiders ( $P < 0.01$ ). A separate two-way (group  $\times$  mPFC region) ANOVA of data only from the stressed groups (data normalized to controls) revealed a significant interaction effect on pERK,  $F(1,64) = 5.55$ ,  $P < 0.05$ , and post-hoc analyses indicated that normalized pERK levels were significantly higher in vmPFC versus dorsomedial PFC (dmPFC) of Avoiders ( $P < 0.05$ ). As illustrated in Figure 2b, a two-way (group  $\times$  amygdala region) ANOVA of data only from the stressed groups (data normalized to controls) revealed a significant main effect of region on pERK,  $F(1,63) = 8.15$ ,  $P < 0.01$ , which indicated that Avoider and Non-Avoider groups exhibited collectively higher pERK expression in the central nucleus of the amygdala (CeA) versus basolateral amygdala (BLA). In accordance with subgroup data, avoidance of the odor-paired environment was significantly correlated with vmPFC pERK levels ( $r^2 = 0.17$ ,  $P < 0.05$ ). We next determined within-subject, inter-regional correlations of ERK phosphorylation upon re-exposure to the predator odor-paired context (Figure 3). Significant correlations were found between BLA and CeA in both Avoiders ( $r^2 = 0.46$ ,  $P < 0.01$ ) and Non-Avoiders ( $r^2 = 0.42$ ,  $P < 0.05$ ), but not in stress-naive controls ( $r^2 = 0.19$ ,  $P = 0.21$ ). Furthermore, in Avoiders, but not in Non-Avoiders or Controls, there was a significant correlation between dmPFC and BLA ( $r^2 = 0.68$ ,  $P < 0.01$ ) and a marginally non-significant correlation between vmPFC and CeA ( $r^2 = 0.20$ ,  $P = 0.06$ ). In most cases, inter-regional correlations were bidirectional, that is, correlative data comprised both increases and decreases in ERK phosphorylation.



**Figure 3.** Within-subject between-region correlations of ERK phosphorylation upon re-exposure to the predator odor-paired context. **(a)** In Avoiders (left panel), but not in Non-Avoiders (right panel) or Controls (data not shown), there was a significant correlation between dorsomedial prefrontal cortex (dmPFC) and basolateral amygdala (BLA) ERK phosphorylation (pERK) expression. Panel **b** reports the strength and direction of correlations between pERK expression in ventromedial PFC (vmPFC) and central nucleus of the amygdala (CeA; top row), and also between BLA and CeA (bottom row) in Avoiders, Non-Avoiders and Controls upon re-exposure to predator odor-paired context.



**Figure 4.** Relationship between alterations in individual levels of alcohol drinking (19 days post conditioning) and odor-paired-context-induced ERK phosphorylation in the dorsomedial prefrontal cortex (dmPFC) and basolateral amygdala (BLA). Elevations in drinking after odor exposure significantly correlated with context-induced reductions in ERK phosphorylation (pERK) levels in both the dmPFC **(a,  $r^2=0.25, P<0.05$ )** and BLA **(b,  $r^2=0.24, P<0.05$ )** of Avoiders, but not of Non-Avoiders or Controls.

#### Elevated drinking levels correspond to traumatic context-associated ERK phosphorylation

Finally, we examined individual relationships between alterations in ethanol drinking behavior and biochemical reactivity to the stress-paired context. Elevations in ethanol self-administration after predator odor exposure were significantly and negatively correlated with context-induced pERK levels in both the dmPFC ( $r^2=0.25, P<0.05$ ) and BLA ( $r^2=0.24, P<0.05$ ) in Avoiders (Figure 4). In contrast, weak positive correlations ( $r^2=0.13–0.15$ ) were observed between post-odor drinking and ERK phosphorylation in these two regions in Non-Avoiders.

#### DISCUSSION

PTSD is a complex psychiatric disorder that is highly comorbid with alcohol-use disorders, although the underlying neural and

molecular perturbations have not been identified. In the present study, animals that persistently avoided a traumatic context exhibited persistent increases in alcohol drinking over weeks. This result supports the notion that traumatic stress reactivity is associated with increased risk for alcohol-use disorders. It is important to emphasize that drinking tests in these experiments occurred in a separate and distinct environment from the traumatic stress experience, consistent with the human PTSD situation. Previous studies have used avoidance as an index for dividing subpopulations of stressed rodents (for example, see Russo *et al.*<sup>26</sup>), and in our study the animals that displayed avoidance of the odor-paired context (Avoiders) did so persistently, consistent with previous findings from other labs.<sup>27</sup> Further, a single odor exposure was sufficient to elevate drinking in Avoiders for at least 19 days, and avoidance behavior across individual animals was predictive of alcohol drinking 19 days later.

Finally, Avoiders exhibited compulsive-like alcohol drinking relative to Non-Avoiders and unstressed controls, as evidenced by the attenuated ability of quinine adulteration to reduce alcohol drinking in Avoiders.

Re-exposure to the odor-paired environment produced a bidirectional modulation of pERK levels in the vmPFC in stressed groups, with Avoiders displaying higher ERK phosphorylation compared with that of Non-Avoiders. Although neither stress-paired group differed from unstressed (and unselected) controls at the group level, avoidance of the stress-paired environment was predictive of context-driven vmPFC ERK phosphorylation levels (Figure 2c). These data appear to reflect the critical nature of individual sensitivity to traumatic stress-associated contextual challenges. Hyperactivation of the vmPFC is associated with a negative effect<sup>28,29</sup> and major depressive disorder,<sup>30,31</sup> including antidepressant responsiveness.<sup>32</sup> War veterans with vmPFC damage are less likely to develop PTSD;<sup>31</sup> some humans with PTSD exhibit heightened vmPFC activity;<sup>33,34</sup> and in healthy humans, increased blood flow in the vmPFC is predictive of self-reported anxiety during an anticipatory anxiety task.<sup>35</sup> Nevertheless, there is also a large human imaging and animal literature that links PTSD-like symptom provocation with vmPFC hypoactivity.<sup>36</sup> Differences across human imaging studies may be explained by functionally distinct subdivisions of the human vmPFC and/or the use of symptom provocation in some studies (for review, see Myers-Schulz and Koenigs<sup>37</sup>). It is important to note that in our study the vmPFC was defined as the infralimbic cortex plus the ventral portion of the prelimbic cortex, whereas the dmPFC was defined as the dorsal portion of the prelimbic cortex plus the anterior cingulate. A limitation of the current analysis is that the neuronal (and even cellular) phenotypes associated with observed changes in ERK phosphorylation are unknown. This limits functional interpretations of ERK phosphorylation changes in the mPFC, which comprises both excitatory and inhibitory neuronal elements. Nevertheless, the between-region correlations presented here likely reflect a second-order connectivity between subregions of the mPFC and amygdala (see Figure 3).

Animal studies report elevated pERK in vmPFC (but not dmPFC) dendrites after repeated, but not acute, footshock stress in rats,<sup>38</sup> suggesting that multiple exposures to stress and/or stress-paired stimuli may differentially alter the balance of vmPFC plasticity in stress-resilient versus susceptible populations. A critical question is whether this plasticity contributes to the persistent behavioral dysregulation seen in individuals with high stress reactivity. In support of this hypothesis, re-exposure to a context previously paired with footshock triggers vmPFC activation (as measured by c-Fos levels) that is partly predictive of a freezing behavior in that context 28 days (but not 1 day) following the initial stress.<sup>39</sup> The ERK/mitogen-activated protein kinase signaling cascade activates numerous transcription factors, including c-Fos<sup>40</sup> and cAMP response element-binding protein,<sup>41</sup> and is thereby believed to promote long-term memory maintenance.<sup>42</sup> In our study, high avoidance and excessive alcohol drinking were observed in the same rats over weeks, and context-induced ERK phosphorylation in the vmPFC was also observed weeks after conditioning and was predictive of context avoidance, which suggests that ERK interacts with downstream transcriptional mechanisms to mediate persistent behavioral effects of traumatic stress. Similar to the results reported here, vmPFC ERK phosphorylation is associated with enhanced cocaine-seeking following protracted withdrawal periods.<sup>43</sup>

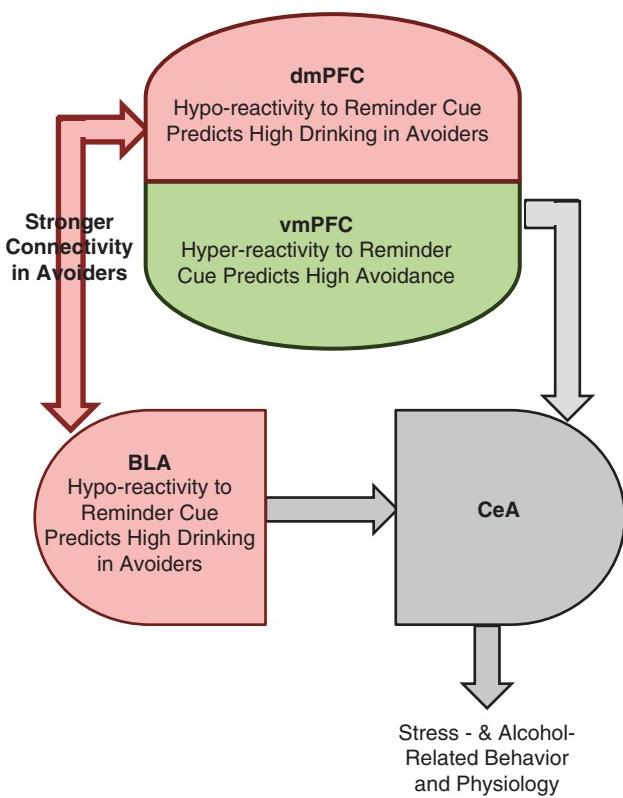
PTSD patients exhibit a strong synchronization of the amygdala and the dmPFC/anterior cingulate cortex upon symptom provocation.<sup>44</sup> Here we show much stronger pERK correlations between dmPFC and BLA in Avoider rats relative to Non-Avoiders and controls (Figure 3). In rodents, intra-BLA infusions of the  $\beta$ -adrenoceptor agonist clenbuterol enhance avoidance memory

and increase plasticity-related proteins (that is, Arc and CaMKII $\alpha$ ) in the dmPFC, whereas BLA inactivation reduces levels of these signaling proteins in the dmPFC.<sup>45</sup> In accordance with our own findings, this avoidance memory-enhancing intra-BLA infusion of clenbuterol reduces BLA ERK phosphorylation.<sup>46</sup> We also found that the odor context-associated changes in ERK phosphorylation in the BLA and CeA were positively and strongly correlated in both groups of stressed rats, but not in stress-naïve controls, consistent with the role of this circuit in anxiety.<sup>47</sup> These data also provide validation for our measure of stimulus-driven neuronal activity intended to model symptom provocation studies in PTSD subjects. Increased activation of the CeA, the major output region of the amygdala, in stressed rats likely drives downstream effector regions (for example, the hypothalamus and periaqueductal gray) responsible for mediating stress- and alcohol-related behavioral and physiological processes.<sup>48</sup>

Within-subject biochemical measurements have been used to assess regional activation patterns associated with avoidance memory (for example, Holloway-Erickson et al.<sup>45</sup> and McIntyre et al.<sup>49</sup>). Here we show that avoidance of a stress-paired environment associates with a modification of the mPFC–amygdala signaling as assayed by context-induced ERK phosphorylation. Interestingly, most of our correlative data comprise both increases and decreases in pERK, suggesting elaborate changes in connectivity between the mPFC and amygdala. The vmPFC projects to the CeA<sup>50</sup> and the CeA drives both the peripheral and central stress systems,<sup>51</sup> highlighting the importance of this circuitry in stress-related disorders. Both ‘top-down’ models, in which the vmPFC hypoactivity disinhibits stress-associated limbic circuitry (for example, the amygdala<sup>28,37</sup>), and ‘bottom-up’ models, in which the amygdala hyperactivity drives PTSD symptomatology (for example, Rauch et al.<sup>52</sup>), have been proposed. Numerous other PTSD symptom provocation studies suggest the importance of altered amygdala–mPFC connectivity<sup>44,53,54</sup> in accordance with the design and results of the current study. It should be noted that the vmPFC also regulates behavioral and physiological responses to stress by activating the hypothalamic–pituitary–adrenal axis,<sup>55,56</sup> and circulating glucocorticoids sensitize CeA activity,<sup>57–59</sup> resulting in a functionally positive CeA–hypothalamic–pituitary–adrenal feedback loop that promotes long-term stress-related pathology.<sup>60</sup>

As a functional correlate linking stress-induced plasticity and alcohol drinking, context-induced reductions in both the dmPFC and BLA ERK phosphorylation were strongly predictive of post-conditioning increases in alcohol drinking in Avoiders (Figure 4). This relationship was dampened and inverted in Non-Avoiders, implying a distinct relationship between stress reactivity and drinking between these two populations. As mentioned above, the  $\beta$ -adrenoceptor activation may mediate reductions in BLA ERK activity and drive stress-induced escalation of drinking in the Avoider population, whereas the Non-Avoider population may be protected from this mechanism. Support for this hypothesis is provided by evidence that propranolol reduces excessive, but not moderate, alcohol drinking in rats.<sup>61</sup> The ERK/mitogen-activated protein kinase signaling cascade is also linked with other stress-related receptors in the limbic system, particularly  $\alpha$ -1 adrenergic receptors<sup>62</sup> and CRF1Rs.<sup>63</sup> Antagonism of both  $\alpha$ -1 adrenergic receptors and CRF1Rs reduces excessive alcohol drinking and other dependence-related behaviors in animals.<sup>64–67</sup> Indeed, the brain norepinephrine systems are being investigated as a therapeutic target for PTSD.<sup>68–70</sup>

Collectively, our data suggest unique alterations of the mPFC–amygdala signaling in Avoider rats (Figure 5) that drive persistent, excessive and compulsive-like alcohol drinking. Going forward, this model will be useful for elucidating the neurobiology of enhanced vulnerability for alcohol-use disorders in humans with PTSD, and may provide valuable targets for behavioral and pharmacological intervention into these oft co-occurring disorders.



**Figure 5.** Proposed model of corticolimbic neurocircuitry underlying traumatic stress-induced avoidance and increases in excessive alcohol drinking. Traumatic stress reminder cue-induced neuronal activation (that is, ERK phosphorylation (pERK) expression) in the ventromedial prefrontal cortex (vmPFC) predicts high avoidance behavior. Also depicted here is that the hyporeactivity of the dorsomedial PFC (dmPFC) and basolateral amygdala (BLA) to a traumatic stress reminder is highly predictive of each other and is also highly predictive of escalated post-stress alcohol drinking only in Avoider rats, suggesting an increased dmPFC-BLA connectivity in Avoider rats. Two potentially important aspects of this circuitry are not depicted here: (1) the population of intercalated  $\gamma$ -aminobutyric acid (GABA) cells that act as a relay for some but not all central nucleus of the amygdala (CeA) afferents, and (2) the neuroendocrine (hypothalamic–pituitary–adrenal) stress axis that is driven by vmPFC activity and inhibited by dmPFC activity,<sup>55,56</sup> and which itself may interact with the CeA in a reciprocally excitatory loop.<sup>60</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

## ACKNOWLEDGEMENTS

This work was supported by NIH grants AA018400 (NWG), AA020839 (SE), AA006420, AA008459, AA012602 and AA020608 (GFK), and by The Pearson Center for Alcoholism and Addiction Research at TSRI. This is publication number 22051 from The Scripps Research Institute.

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